Detection and Characterization of a New Strain of Citrus Canker Bacteria from Key/Mexican Lime and Alemow in South Florida

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ABSTRACT

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In the Wellington and Lake Worth areas of Palm Beach County, FL, citrus canker appeared on Key/Mexican lime (*Citrus aurantiifolia*) and alemow (*C. macrophylla*) trees over a period of about 6 to 7 years before detection, but nearby canker-susceptible citrus, such as grapefruit (*C.* × *paradisi*) and sweet orange (*C. sinensis*), were unaffected. Colonies of the causal bacterium, isolated from leaf, stem, and fruit lesions, appeared similar to the Asiatic group of strains of *Xanthomonas axonopodis* pv. *citri* (*Xac*-A) on the nutrient agar plate, but the growth on lima bean agar slants was less mucoid. The bacterium produced erumpent, pustule-like lesions of typical Asiatic citrus canker syndrome after inoculation into Key/Mexican lime, but brownish, flat, and necrotic lesions on the leaves of Duncan grapefruit, Madame Vinous sweet orange, sour orange (*C. aurantium*), citron (*C. medica*), Orlando tangelo (*C. reticulata* × *C.* × *paradisi*), and trifoliate orange (*Poncirus trifoliata*). The bacterium did not react with the *Xac*-A specific monoclonal antibody A1 using enzyme-linked immunosorbent assay (ELISA) and could not be detected by polymerase chain reaction (PCR)-based assays using primers selected for *Xac*-A. DNA reassociation analysis confirmed that the pathogen, designated as *Xac*-A^{W}, was more closely related to *Xac*-A and *Xac*-A* strains than *X. axonopodis* pv. *aurantifolii* or the citrus bacterial spot pathogen (*X. axonopodis* pv. *citrumelo*). The strain can be easily differentiated from *Xac*-A and *Xac*-A* using ELISA, PCR-based tests, fatty acid analysis, pulsed-field gel electrophoresis of genomic DNA, and host specificity.

Additional keywords: canker eradication, disease diagnosis, taxonomy of xanthomonads

Asiatic citrus canker (ACC), caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. campestris* pv. *citri*) (*Xac*-A), is a serious disease of many commercial citrus varieties and some citrus relatives. The bacterium produces a unique syndrome ranging from pustules to necrotic lesions consisting of erumpent corky tissues surrounded by water-soaked tissues and a yellow halo on leaves, stems, and fruit (8,36). With training and practice, a citrus canker diagnostician is able to visually identify ACC in the field. Severe ACC on a susceptible variety results in defoliation, twig dieback, premature fruit drop, and blemished fruit that consequently reduce fruit production and its market value due to consumer preference. Shipment of fruit from an infested area to a disease-free citrus production region is prohibited by regulations (36).

Other types of citrus canker–causing bacteria have also been detected, diagnosed, and characterized over the last 20 years using pathogenicity, biochemical, physiological, serological, and molecular methods (2,9,11,12,14,17,19,22,24,25,32–34,40,41,43,47–51). Classification of canker strains within the genus *Xanthomonas* has been discussed extensively (18,32,35,38,44–47,52–54), and several distinguishable groups of citrus canker–causing bacteria have been recognized under the species *axonopodis*. Cancrosis B, or false canker (formerly known as B-strain canker), was discovered on lemon (*Citrus limon*) in Argentina in 1923 (8). The disease occurred primarily on *C. limon* and *C. aurantiifolia*, but also affected *C. aurantium*. Key/Mexican lime cancrosis (formerly known as C-strain canker) was reported in Brazil on Key/Mexican lime (*C. aurantiifolia*) in 1963. The causal bacteria of the latter diseases that produce a very similar canker syndrome on their limited citrus hosts are genetically related to each other but different from *Xac*-A and therefore have been referred to as strains of *X. axonopodis* pv. *aurantifolii* (*Xaa*) (18). Recently, Vernière et al. designated some strains restricted to Key/Mexican lime as *Xac*-A* based on their physiological and genetic similarities and serological difference from *Xac*-A (49), indicating that heterogeneous strains of citrus canker–causing bacteria may be placed under pathovar *citri.*

Citrus bacterial spot (CBS), formerly known as Florida nursery strain citrus canker or E-strain canker (18,21,39,41), is caused by *X. axonopodis* pv. *citrumelo*. This disease differs from citrus canker by producing flat necrotic spots with a yellow halo on leaves and twigs and rarely on fruit. CBS occurs almost exclusively in nurseries and has been reported on many citrus varieties, including grapefruit, sweet orange, mandarin, tangerine, sour orange, lemon, and Key/Mexican lime. The pathogen differs genetically from the other xanthomonads causing citrus canker (14).

Since 1984 when CBS was found in Florida citrus nurseries, considerable information has been accumulated, allowing the differentiation of the various taxa of citrus canker–causing and other similar citrus bacterial pathogens. Pathogenicity tests (41), physiological analyses (5,43,46,48), serological tests using antibodies (1,2,5,9,19), fatty acid analyses (34,40,47), total protein profiles (47), plasmid DNA patterns (33), plasmid-based hybridization probes (21,47), polymerase chain reaction (PCR)-based assays (10–12,25), restriction enzyme analyses of amplified DNA fragments of a *hrp*-related DNA sequence (14),

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and DNA-DNA hybridizations (47) have been used for differentiating these bacteria. The Florida Department of Agriculture and Consumer Services (FDACS)-Division of Plant Industry (DPI)-Plant Pathology Quarantine facility in Gainesville has used pathogenicity tests routinely to confirm Acc on the samples from the infected citrus first found in a square mile. The commercial *Xac*-A specific monoclonal antibody, A1 for ELISA, and specific *Xac*-A primers (25) are used for a quick identification of ACC-causing bacteria in order to expedite the process of removing infected trees and all citrus that is located within a radius of 1,900 feet (579 m).

Since 1986, there have been two major outbreaks of A-strain citrus canker in Florida, one in the commercial area of Tampa Bay and another in the residential area of Miami-Dade County (20,36). Discerning the strains from two different locations and knowing their possible association to a new CC outbreak has become an issue for the regulators with the Citrus Canker Eradication Program (CCEP) to track down the possible route of disease spread. Recently, a rep-PCR based technique with BOX and ERIC primers has been developed to discern not only all pathotypes of CC-causing bacteria worldwide but also subgroups under pathotype *citri* associated with certain geographic areas of the world (10,12). Two genotypes, Manatee and Miami, were thus identified, and *Xac*-A bacteria were believed to have been introduced into Florida separately approximately 2 years prior to 1986 and 1995, respectively (10,36). Since then, the technique has been used to determine the genotype of strains obtained from CC samples collected from a new outbreak.

During a routine survey for ACC in Palm Beach County, FL, in May 2000, typical ACC symptoms were observed on leaves, stems, and fruit of many Key/Mexican lime and alemow (*Citrus macrophylla* Wester) trees (Fig. 1A to C) located in the vicinity of the cities of Wellington and Lake Worth, where ACC had not been previously detected. Other ACC-susceptible citrus trees near the infected ones (within 20 m of a diseased tree) were not affected (unpublished data). Field disease evaluation and initial laboratory tests revealed that the causal bacterium did not produce ACC symptoms on Duncan grapefruit after inoculation and that monoclonal antibody A1 did not combine with the bacterium in ELISA. In this study, we characterized the strains, which had differential host specificity for Key/Mexican lime and alemow trees, using phenotypic and genetic techniques and placed them in the proper context in relation to other xanthomonads pathogenic to citrus. We present conclusive data that these strains with unique host specificity differ significantly from other *Xac*-A strains and therefore have designated them as the "Wellington" strain (*Xac*-A^W).

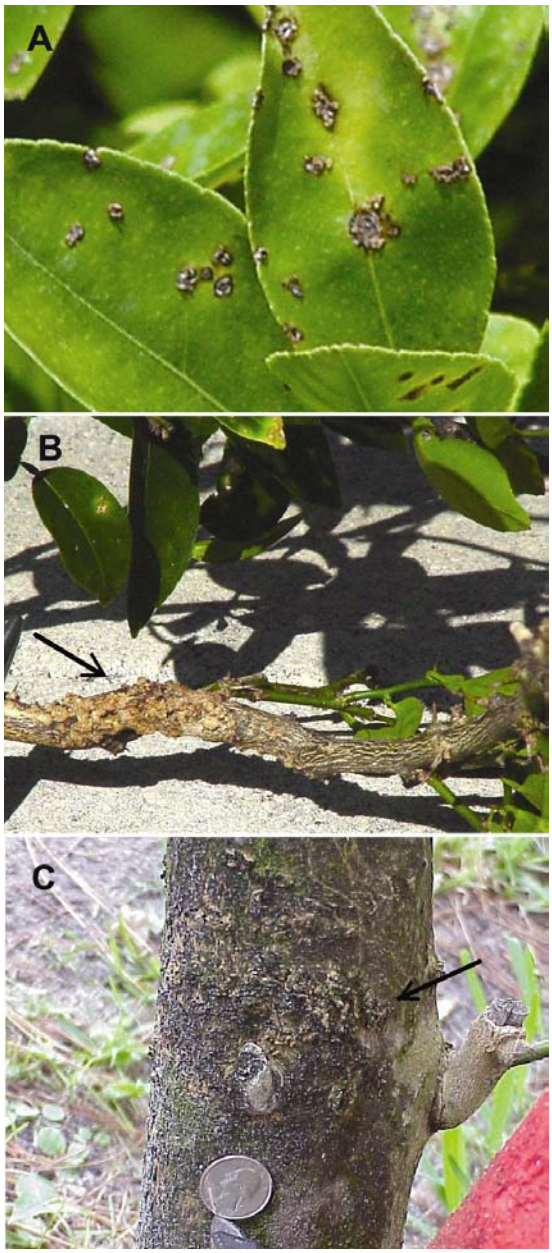

Fig. 1. Canker symptoms caused by *Xanthomonas axonopodis* pv. *citri Xac*-A^W strain citrus canker bacterium on **A,** leaves and **B,** twig of Key/Mexican lime, and **C,** on the trunk of a 6- to 7-year-old alemow tree.

MATERIALS AND METHODS

Bacterial isolation and pathogenicity tests. All citrus canker–causing bacteria were isolated by crushing canker tissue in sterile tap water, streaking the macerate on nutrient agar (NA) plates, and picking up the single colonies 3 days after incubation at 32°C. The suspensions from crushed lesions were also infiltrated by syringe into young Duncan grapefruit and Key/Mexican lime leaves. Lesions appearing 4 to 6 days after those inoculations also were used for isolation of the pathogen if attempts to isolate the bacterium from fresh samples failed. Most of the isolated *Xac*-A^W strains were further tested for their pathogenicity on Key/Mexican lime and Duncan grapefruit at concentrations of 10^8 and 10^3 CFU/ml, and for their hypersensitive reaction (HR) on tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum* var. *annuum*), and tobacco (*Nicotiana tabacum*) at concentrations of 10^8 CFU/ml. All inoculated plants were kept in the greenhouse within the DOACS-DPI quarantine facility with an average temperature of 26°C. The cultures of isolated bacteria were purified and stored on lima bean agar (LBA) slants, in sterile tap water, and in 20% glycerol at –80°C. All strains used in this investigation are listed in Table 1.

Inoculation experiments. Each LBA culture of selected isolates was diluted to approximately 10^8 and 10^3 CFU/ml using sterile tap water. Bacterial suspensions

were infiltrated into three to five leaves of Duncan grapefruit, rough lemon, Eureka lemon, Key/Mexican lime, sour orange, sweet orange, tangelo, trifoliate orange, and citron in the quarantine greenhouse. The inoculated plants were kept in the greenhouse and examined daily for canker symptoms for 1 month and then destroyed. Four *Xac*-A and four *Xac*-A^W cultures were used as replications on a small plant of each citrus species for the experiment. Three *Xac*-A^* strains and two CBS bacterial cultures were used as comparisons.

Field experiments. Two small greenhouse-grown plants (approximately 50 to 80 cm tall) of grapefruit, rough lemon, Eureka lemon, Key/Mexican lime, sour orange, and Madame Vinous sweet orange were placed under the canopy of a 7-year-old, naturally occurring alemow tree heavily infected with *Xac*-A^W and located in the Wellington area. The plants were examined weekly in the field for 6 weeks and then transported to the Plant Pathology Quarantine facilities of FDAC-DPI in Gainesville for observation of symptoms and isolation of the bacterium. Bacterial cultures from each symptomatic citrus plant were collected and tested for pathogenicity on Key/Mexican lime.

In vivo population dynamics. Potted greenhouse-grown Key/Mexican lime and Duncan grapefruit plants were pruned to produce uniformly aged shoots. Three young shoots were maintained on each plant. A single colony of *Xac*-A^W (X0012875), *Xac*-A (X0012878), and *Xac*-A^* (XC-201) was transferred to nutrient broth. Bacteria in the log-phase of growth were pelleted by centrifugation, resuspended in sterile tap water, and adjusted to 10^8 CFU/ml (0.3 OD reading at 600 nm). A 10^5 CFU/ml suspension was obtained by dilution of suspension at 10^8 CFU/ml in sterile tap water. Each bacterial suspension was infiltrated into each of 15 leaves, using a 1-ml syringe and 25-gauge needle. Inoculated plants were kept in a quarantine greenhouse at 20 to 30°C. Infiltrated leaves were sampled 0, 1, 2, 4, and 8 days after infiltration. At each sampling period, two leaf disks (about 1 cm^2 total) from infiltrated tissue were triturated in sterile tissue grinders containing 1 ml of sterilized tap water. The suspension was serially diluted 10-fold, and 100 µl of developed dilutions were spread onto nutrient agar plates. Three infiltrated leaves were used as replications for each time interval. The colonies on each plate with most readable numbers (5 to 150 colonies per plate) were counted after the plates were incubated at 28°C for 72 h. The data were analyzed with SAS general linear model (GLM) procedure, and a population growth curve was plotted with means at different sampling intervals.

Metabolic profiles. Pure cultures of selected strains were grown and tested for utilization of the 95 carbon sources available on the GN Microplate (Biolog Inc., Hayward, CA) as previously described (4). The carbon utilization patterns were read with a microplate reader and analyzed by a cluster analysis program provided by Biolog.

ELISA. Selected strains were grown on LBA slants for 2 days and tested against monoclonal antibody A1 selected by Alvarez et al. (1) and commercialized by Agdia Inc. (Elkhart, IN). A loopful of a bacterial culture from an LBA slant was suspended in 3 ml of sterile tap water. The suspensions were tested using the standard protocol provided by Agdia.

Fatty acid (FAME) analysis. Fatty acid profiles were generated for selected strains. All bacterial cultures were grown on trypticase soy broth agar (BBL Laboratories, Cockeysville, MD) at 28°C for 24 h. Whole-cell fatty acid methyl esters

Table 1. *Xanthomonas* cultures used in this study

No. of isolates	Isolate designations	Group[a]	Host	Origin	Source
14	X0012867, X0012873, X0012875, X0012881, X0012883, X0012884, X0000052, X0000053, X0000054, X0000055, X0000057, X0000058, X0000063, X0000103	*Xac*-A^W	*Citrus aurantiifolia*	Wellington & Lake Worth, FL, USA	DPI[b]
2	X0012881, X0000062	*Xac*-A^W	*C. macrophylla*	Wellington, FL, USA	DPI
8[c]	X00W1, X00W2, X00W3, X00W4, X00W5, X00W6, X00W7, X00W8	*Xac*-A^W	*C. aurantiifolia*	PPQF green house, Gainesville, FL, USA	DPI
6	XC270, XC280, XC290, XC322, XC406, XC205	*Xac*-A^*	*C. aurantiifolia*	Southwest Asia	USDA[d]
13	X0012876, X0012877, X0012878, X0012879, X0012885, X9700054, X9803195, X9912777, X0012855, X0012834, X0012839, X0013042, X9905582	*Xac*-A	*Citrus* spp.	Miami, FL, USA	DPI
6	XI9900001, XI9900002, XI0000157, XI0000075, XI0000158, XI0000159	*Xac*-A	*Citrus* spp.	Immokalee, FL, USA	DPI
8	X0000064, X0000065, X0000066, X0000067, X0000068, X0000069, X0000070, X0000071	*Xac*-A	*C. aurantiifolia*	Northern Miami, FL, USA	DPI
11	ATCC49118, XS99-65, XS99-82, XS99-97, M2, M4, M13, M14, M16, X9601269, X9601713	*Xac*-A	*Citrus* spp.	Manatee Co., FL, USA	DPI
1	B-69	*X. axonopodis* pv. *aurantifolii*	*C. limon* Lemon	Argentina	UF[e]
1	ATCC51306	*X. axonopodis* pv. *aurantifolii*		Florida, USA	ATCC[f]
1	ATCC51302	*X. axonopodis* pv. *aurantifolii*		Florida, USA	ATCC
1	P99001283	*X. axonopodis* pv. *citrumelo*	*Citrus* sp.	Avon Park, FL, USA	DPI
1	X0012862	*X. axonopodis* pv. *citrumelo*	*C. aurantium* Sour orange	Boca Raton, FL, USA	DPI
1	XS9900061	*X. axonopodis* pv. *citrumelo*	*C.* × *paradisi* Grapefruit	Zellwood, FL, USA	DPI
1	ATCC49120 (XCC3048)	*X. axonopodis* pv. *citrumelo*	*Citrus* sp.	Florida, USA	ATCC
1	ATCC35938	*X. axonopodis* pv. *vasculorum*	*Saccharum officinarum*		ATCC

[a] Strains of xanthomonads used.
[b] Plant Pathology Quarantine Facility, Division of Plant Industry (DPI).
[c] Pure culture was obtained from lesions on the Key/Mexican lime plants inoculated with different A^W isolates from the Wellington area.
[d] USDA-ARS.
[e] University of Florida.
[f] American Type Culture Collection.

(FAMEs) were extracted and characterized as described previously (22). All numerical analyses for the FAME dendrogram were performed with the Microbial Identification System (MIS) software (version 3.60, Microbial ID, Newark, DE). Profiles were pooled and subjected to the Principal Components Analysis using the software with the MIS to determine relationships among strains.

Polymerase chain reaction (PCR)-based assay. Bacterial cultures were grown in Luria broth (LB) for 14 to 16 h at 28°C. DNA was purified using the CTAB method (3). DNA was precipitated overnight at –20°C with isopropanol and finally washed with 70% ethanol. After drying, the pellet was resuspended in 25 µl of DNase free water and the two aliquots combined for the PCR assay.

PCR was performed with a heated lid Peltier thermocycler (PTC-200, MJ Research,Watertown, MA). The primers (5′ TGT CGT CGT TTG TAT GGC 3′ and 5′ GGG TGC GAC CGT TCA GGA 3′) amplified a 468-bp fragment of plasmid DNA specific to *Xac*-A (25). The amount of DNA template utilized for the final PCR reaction was optimized by titration and thus variable. The reaction mixture was comprised of 1.0 unit of *Taq* polymerase (Fisher Scientific, Fair Lawn, NJ), primers at 10 pM (synthesized by Life Technologies Inc., Gaithersburg, MD), dNTPs at 200 µM each (Boehringer Mannheim, Mannheim, Germany), reaction buffer containing 10 mM Tris-HCl and 50 mM KCl (Fisher Scientific) and $MgCl_2$. The latter was optimized by titration with a final Mg^{++} concentration of 2.0 mM being utilized. The thermocycler was preheated to 70°C before sample loading and programmed as follows: initial denaturation at 94°C for 2 min followed by 34 cycles of denaturation at 94°C for 10 min, annealing at 50°C for 30 s, and extension at 72°C for 1 s. The final extension proceeded for 10 min at 72°C and a final incubation at 4°C. The PCR-amplified products were separated on a 1.5 to 2.0% agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, and imaged.

Pulsed-field gel electrophoresis. The methods described by Egel et al. (14) were followed. The DNA derived from selected bacterial strains (Table 1) was digested with *Xba*I or *Spe*I (Boehringer Mannheim) restriction enzymes. The DNA fragments were separated in a CHEF DRII unit (Bio-Rad Laboratories, Richmond, CA). The resulting large DNA fragments were separated by pulsed-field electrophoresis. The gels were stained with ethidium bromide and photographed with type 55 Polaroid film. All strains were run on the same gel, and similarity coefficients were determined for strains by comparing fragments greater than 100 kb. Similarity values were calculated as described by Egel et al. (14), by using the mathematical equation proposed by Nei and Li (31) based on the proportion of shared DNA fragments. The number of nucleotide substitutions per site was estimated by the iterative method of Nei (30) by using the SAS program as described by Leite et al. (29). The KITSCH program from the PHYLIP computer package was used to infer a rooted phylogenetic tree by using the Fitch-Margoliash method (15,16). The input data consisted of a matrix of pairwise genetic distances determined as estimates of the numbers of nucleotide substitutions per site on the basis of similarity coefficients calculated for the digestion data of the enzymes.

Table 2. Characterization of unique *Xanthomonas axonopodis* pv. *citri* strains (*Xac*) obtained from Key/Mexican lime and alemow in South Florida

Diagnostic protocol	A	A*	A^W	CBS
ELISA (monoclonal antibody A1)				
on fresh lesion	+ (12)[a]	NT[b]	– (22)	– (3)
on pure culture	+ (7)	– (6)	– (7)	– (3)
PCR identification 6/7 and 4/7				
on pure culture	+ (18)	+ (3)	– (17)	– (10)
Host range tested in greenhouse[c]	(4)	(3)	(4)	(2)
Grapefruit	+	–	–	–
Rough lemon	+	V[d]	V	–
Eureka lemon	+	–	V	–
Key lime	+	+	+	–
Sour orange	+	–	–	–
Sweet orange	+	+	–	–
Tangelo	+	–	–	–
Trifoliate orange	+	–	–	–
Citron	+	–	–	–

[a] Number in parentheses indicates number of strains used.
[b] NT: not tested.
[c] + indicates that a typical citrus canker symptom caused by *Xac*-A was present unless explained otherwise. – indicates the absence of such a symptom.
[d] V indicates that lesions were slightly raised, but the epidermis was not ruptured.

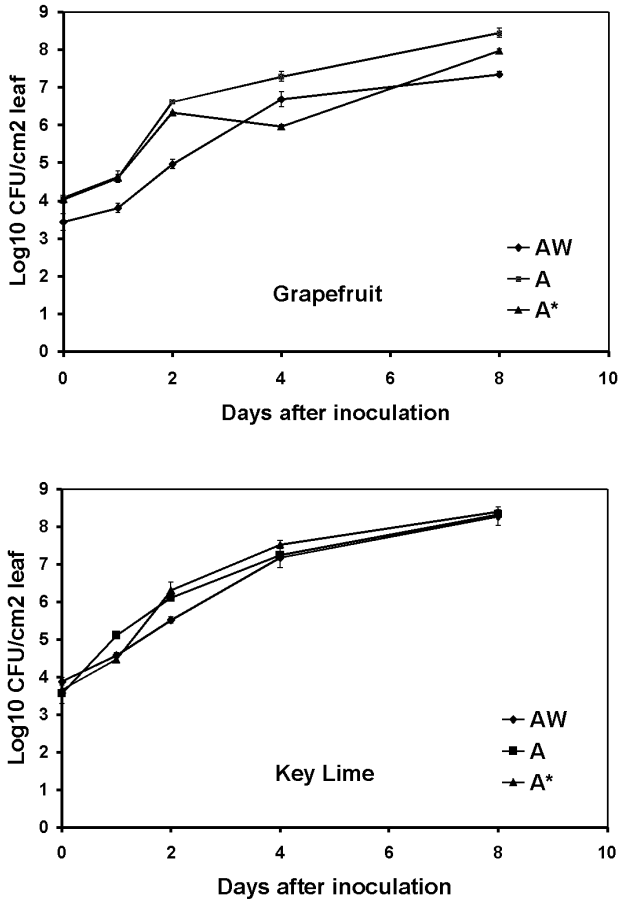

Fig. 2. Population dynamics of *Xanthomonas axonopodis* pv. *citri Xac*-A^W strain in grapefruit and Key/Mexican lime leaves at inoculum level of 10^5 CFU/ml.

DNA reassociation. The S_1 nuclease procedure for the free solution reassociation for DNA similarity assays was used for DNA reassociation of selected strains (Table 1) and is the most robust method for measuring heteroduplex formation as reviewed (26–28,38). All procedures, including DNA isolation, French pressure cell fragmentation of DNA, hybridization, and the S_1 nuclease assays, are detailed by Johnson (28). However, rather than chemically labeling probe DNA with ^{125}I, the random primers method was used (RadPrime Labeling System, Life Technologies, Inc.) to label with alpha-^{33}P dCTP (NEN Life Science Products, Inc., Boston, MA). The probe and target DNAs were reassociated at 67 ± 0.5°C for 24 h. With 22.7% formamide, this is equivalent to reassociation in the absence of formamide at 80.8°C (=T_m 95.8-15°C for xanthomonad DNA at 63.6 to 64.1 G+C%; Bradbury, 1984). Following reassociation, incubation with S_1 nuclease digests ssDNA strands, loops, and ends. Beta emissions from the remaining heteroduplex that has incorporated labeled DNA are estimated by scintillation counter from washed precipitates on glass fiber filters. Percent heterologous reassociation was determined by comparing the radioactivity detected to that obtained from homologous reassociations. Values for both homologous and heterologous reassociations were corrected for nonspecific heteroduplex formation by controls with salmon sperm ssDNA. Each reaction was repeated at least once. An average number was reported as % DNA similarity.

RESULTS

Bacterial isolation. Fifty-six strains of the bacterium were obtained from leaf, stem, and fruit samples collected from symptomatic Key/Mexican lime and alemow plants in the Wellington and Lake Worth vicinities. One strain was recovered from a bark lesion on the trunk of an approximately 6- to 7-year-old alemow tree (Fig. 1). In addition, strains were also recovered from lesions in leaves of the potted citrus plants that were placed under the canopy of a severely diseased alemow tree in the field. The phenotypic characteristics of the strains on NA plates or LBA slants appeared similar to, but not as slimy as, those of *Xac*-A. Like other xanthomonads, colonies were mucoid, convex, and yellow on NA plates. They also produced the unique yellow pigment xanthomonadin and large amounts of extracellular polysaccharide.

Inoculation experiments. Like other strains of *Xac*-A, all *Xac*-A^W strains caused a hypersensitive reaction (HR) on tomato, but not on pepper and tobacco. Both strains produced identical symptoms when suspensions of 10^8 and 10^4 CFU/ml were artificially infiltrated into intercellular spaces of a Key/Mexican lime leaf (Table 2). All *Xac*-A^W strains tested in the experiment did not produce the typical canker symptoms that *Xac*-A strains did on other citrus species. However, on rough lemon and Eureka lemon, the infiltrated areas with the high concentration of cells were flat and necrotic, but slightly raised at the edge of the lesions. The areas of nonhost leaves infiltrated with the highly concentrated *Xac*-A^W bacterium (10^8 CFU/ml) became water-soaked, and chlorosis developed about 4 days after inoculation with no hyperplasia. The inoculated areas collapsed and became necrotic in the center 8 days later. The inoculated leaves of grapefruit, sweet orange, trifoliate orange, and tangelo abscised readily 5 to 10 days after inoculation. The symptoms on these inoculated lemon leaves differed from those inoculated with *Xac*-A at the same cell concentration. Individual canker lesions developed on Key/Mexican lime 7 days after inoculation with low concentrations (10^3 CFU/ml) of *Xac*-A^W, but not on grapefruit leaves. Slight discoloration occurred on some grapefruit leaves 7 to 10 days after inoculation with low concentration of *Xac*-A^W, which, however, disappeared approximately 3 weeks after inoculation.

In the field trial, where naturally infected trees were present, lesions were detected on Key/Mexican lime plants 14 days after they were placed under a heavily diseased alemow tree, and a typical ACC syndrome developed 6 weeks later. Some small and slightly raised lesions with a water-soaked margin (not typical canker lesions of ACC) appeared on several leaves of one rough lemon plant within 17 days, and similar lesions appeared on sour orange, Duncan grapefruit, and rough lemon in 38 days. The causal bacterium was recovered from those lesions, and it was

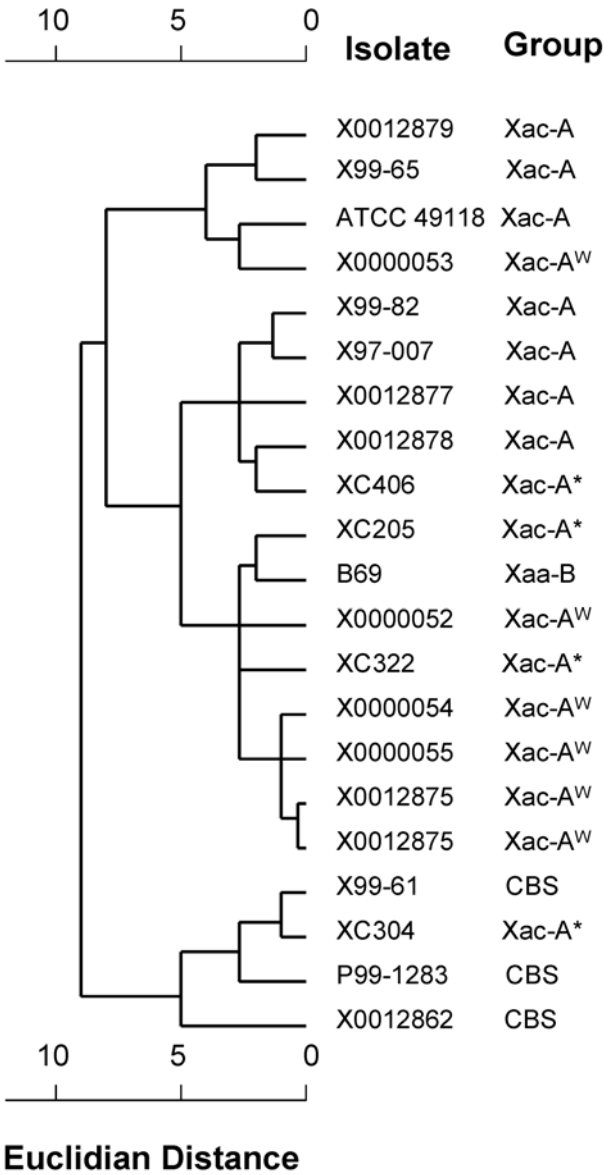

Fig. 3. Dendrogram showing the relationships among *Xanthomonas axonopodis* pv. *citri Xac* strains and citrus bacterial spot (CBS) bacterium based on differential utilization of the 95 carbon substrates available in the Biolog GN Microplate.

confirmed as *Xac*-A^W through discerning symptoms on Key/Mexican lime and grapefruit after inoculation.

In vivo population dynamics. In general, the populations of *Xac*-A^W, *Xac*-A, and *Xac*-A* in Key/Mexican Lime and grapefruit leaves after infiltration with inocula of 10^5 CFU/ml increased over 8 days (Fig. 2). In Key/Mexican lime, no significant difference was observed between different strains at the 5% confidence level (Probability > *F* value = 0.0715), while three strains demonstrated a significant difference in grapefruit (Probability > *F* value = 0.0008). Growth of *Xac*-A^W and *Xac*-A* were obviously slower than growth of *Xac*-A over time in grapefruit (Fig. 2). After day 4, *Xac*-A^W multiplied much more slowly than the other strains. Populations could not be determined after 8 days because leaves became chlorotic and abscised. In leaves of grapefruit plants infiltrated with suspensions of 10^8 CFU/ml of *Xac*-A or *Xac*-A^W, the multiplication pattern of the two strains was similar to that inoculated with 10^5 CFU/ml. However, between 4 and 8 days after inoculation, necrosis occurred in leaves inoculated with *Xac*-A^W, and the populations declined, but not in leaves inoculated with *Xac*-A. The experiment was repeated once, in which 10^8 CFU/ml was infiltrated into leaves and populations were monitored in several grapefruit and Mexican lime plants. Similar results were observed in both experiments. In addition, in a third experiment, populations were monitored following infiltration with 10^5 CFU/ml. Similar results were observed.

Metabolic profiles. The six *Xac*-A^W strains fell into the cluster of *Xac*-A, *Xac*-A*, and *Xaa* strains according to the Biolog metabolic profiles. The CBS strains, along with one *Xac*-A* strain, formed another cluster. None of the genotypes of citrus canker–causing bacteria could be distinguished by metabolic differences based on the Biolog compounds (Fig. 3).

ELISA. The MAb A1 antibody that reacted with all *Xac*-A strains but not with *Xac*-A^W and *Xac*-A* strains in pure culture (Table 2) proved to be useful in differentiating *Xac*-A from *Xac*-A^W and *Xac*-A*. Xanthomonads causing CBS occasionally gave a weak positive as reported previously (2).

Fatty acid (FAME) analysis. *Xac*-A, *Xac*-A^W, *Xac*-A*, and *X. axonopodis* pv. *citrumelo* strains formed distinct clusters, respectively, when principal component 1 was plotted against principal component 2 in multivariate analysis of the fatty acid profiles of each strain (Fig. 4). One *Xac*-A* strain was an outlier. A dendrogram was constructed with representative strains and showed that the *Xac*-A strains were separated from *Xac*-A^W and *Xac*-A* at a Euclidean distance of over 12. The *Xac*-A^W and *Xac*-A* strains were clustered within Euclidean distance of seven units.

PCR assay. DNA amplification did not occur with DNA extracted from any of 17 *Xac*-A^W cultures originating from either diseased trees in the field or from the field-inoculated greenhouse plants using specific primers developed by Hartung et al. (25) for *Xac*-A. Amplification did occur with all *Xac*-A and *Xac*-A* cultures using the same primers (Table 2).

Restriction endonuclease analysis. The clustering of *Xac*-A, *Xac*-A^W, *Xac*-A*, and *Xaa* strains based on genetic differences derived from similarity coefficients of DNA fragments after digestion with *Spe*I is presented in Figure 5A. The clustering of strains on the same basis after DNA digestion with *Xba*I was very similar (Fig. 5B). The *Xac*-A, *Xac*-A*, and *Xac*-A^W strains each formed distinct clusters. However, the

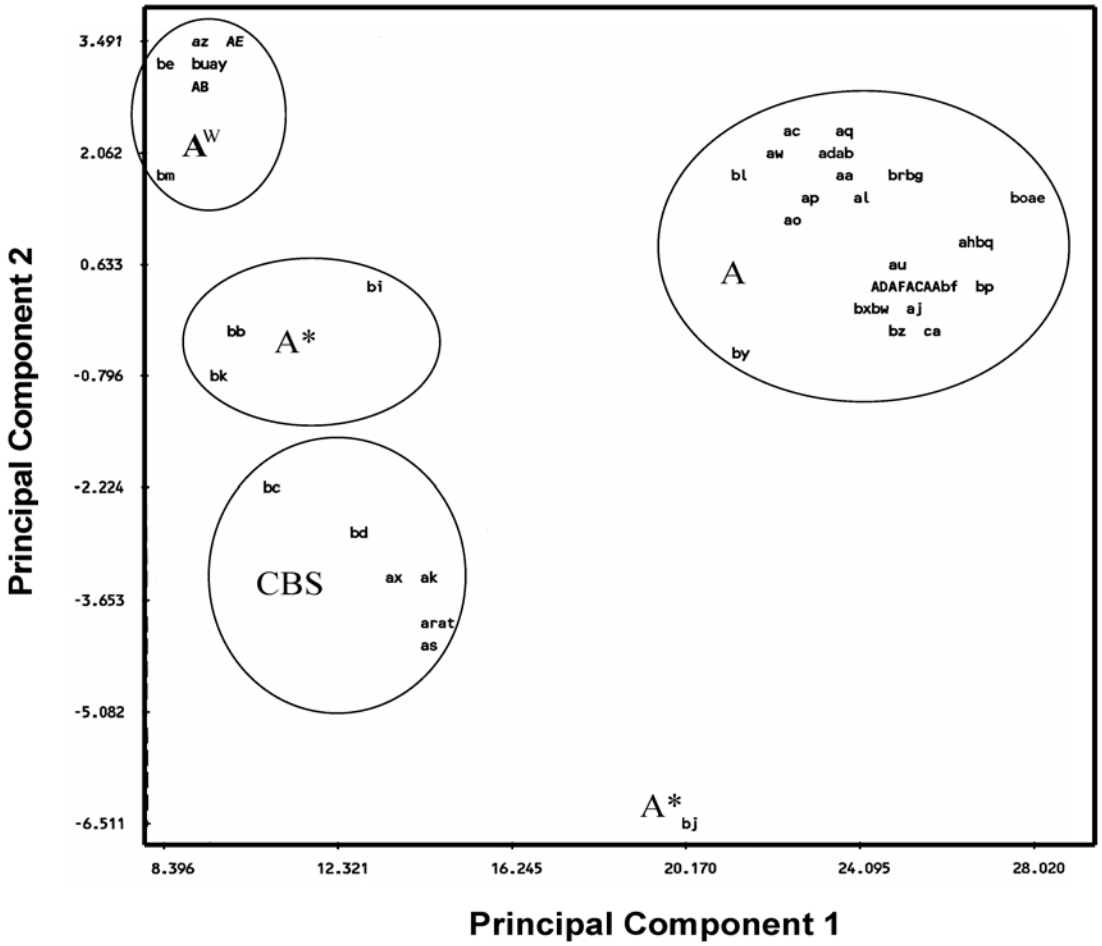

Fig. 4. Principal component analysis of the fatty acid profiles of 34 cultures of *Xanthomonas axonopodis* pv. *citri Xac*-A strain (aa-ax), four cultures of *Xac*-A* (bb, bi-bk), nine cultures of *Xac*-A^W (ay, az, ba, be, bm, bn, and bt-bv), and citrus bacterial spot (CBS) cultures (ak, ar-at, ax, bc, and bd). x-axis: principal component 1, y-axis: principal component 2. AA = ai, af; AB = ba, av; AC = bh, an; AD = bs, am; AE = bt, bn; AF = bw, ag.

Xac-A^W and *Xac*-A* strains were more related to *Xac*-A strains than to the *Xaa* strain.

DNA reassociation. According to the DNA reassociation analysis, all *Xac*-A^W, *Xac*-A*, and *Xac*-A strains were closely related, with DNA similarities ranging from 70.7 to 94.1% (Table 3). They were quite different from strains of *X. axonopodis* pv. *aurantifolii* (34.6 to 50.6% similarities), CBS bacteria (33.3 to 51.7% similarities), and *X. axonopodis* pv. *vasculorum* (13.9 to 25.8% similarities).

DISCUSSION

The work reported here is a polyphasic characterization of a unique strain of citrus canker bacteria that was found on Key/Mexican lime and alemow trees in south Florida in 2000. The strain, unlike the ones causing typical Asiatic citrus canker on most commercial citrus cultivars and some citrus relatives, has a very limited host range in nature. Thorough understanding of the strains and their distribution is of concern to federal and state regulators who must be able to distinguish different strains of xanthomonads pathogenic to citrus by name so that certain regulatory measures can be applied accordingly. Laws for eradication of strains causing Asiatic citrus canker (*X. axonopodis* pv. *citri*) exist in some countries (36), and other xanthomonads pathogenic to citrus are subject to certain other regulations (8,21,36).

All of the unique group of strains used in this study have characteristics of the genus *Xanthomonas* (6) and should be placed in the species *axonopodis* according to the most recent classification scheme (7,37,44,53). In that scheme, percentage of DNA reassociation is primarily used to separate xanthomonads into species. Accuracy and consistency of the DNA similarity test from different laboratories can be very critical in separating the species. Johnson (26,28) and Stackebrandt and Liesack (38) recognized that the different methods of DNA reassociation are comparable. Johnson emphasized that the optical method gave higher values. Stackebrandt and Liesack (38) came closest to stating a preference for the S_1 nuclease system and specifically recommended the methods used by Johnson (26), Brenner et al. (7), and Steigerwalt et al. (42) without mentioning the optical method. While the general relationships detected by optical and S_1 methods are similar, differences are apparent. For instance, the optical method did not discriminate between *X. axonopodis* and ACC-causing bacteria, whereas the S_1 technique does (G. H. Lacy, *unpublished*). That is because the optical method is a more liberal measure of relatedness including measurement of (i) mismatched pairs among matched pairs and (ii) self-hybridization due to the fact that probe and test DNA are each present in equal concentration, so the values must be "corrected" for self-hybridization of the DNA without taking DNA purity into consideration. The S_1 method is more conservative because (i) the probe is present in small percentage in comparison with test DNA (between 1/700 and 1/1,400), which eliminates self-hybridization; (ii) all nonannealed bases are digested by S_1 nuclease and washed away; and (iii) only radioactivity from annealed bases is measured. The method of determining DNA association used in this work is probably more accurate than those reported earlier (14). DNA reassociation data reported here was the primary characteristic used to determine the genetic relatedness of the bacteria causing canker symptoms on citrus, but not used to place the bacteria in proper species.

Differentiation of *Xac*-A^W from *Xac*-A strains has become very important because both strains may coexist in an area, and the regulatory measures pertaining to each

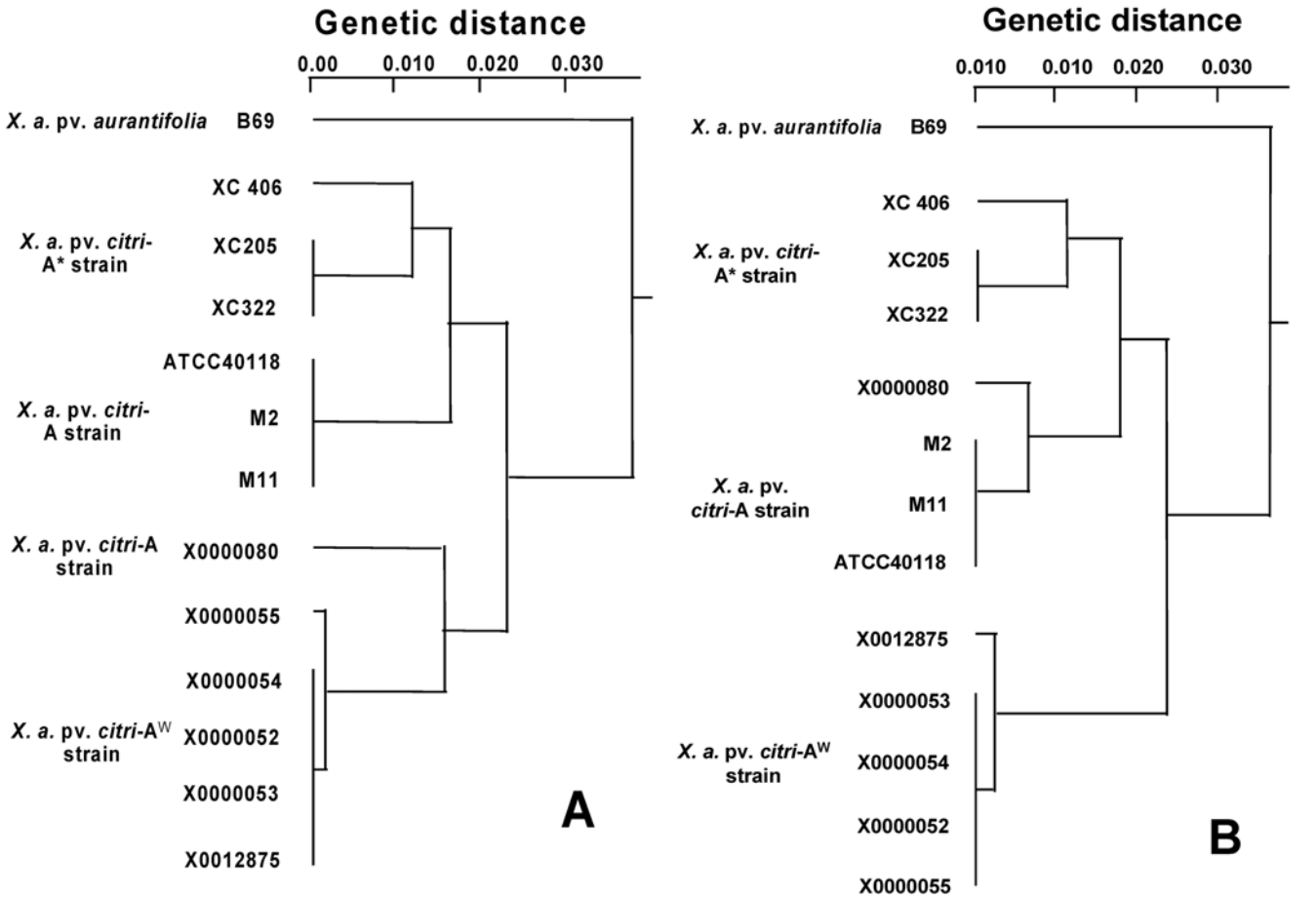

Fig. 5. Clustering of canker-causing bacteria inferred from similarity coefficients obtained from **A,** *Xba*I and **B,** *Spe*I restriction endonuclease digestion data. Tree was generated by KITSH procedure with PHYLIP computer package by using the Fitch-Margoliash method. Genetic distances are estimates of the number of nucleotide substitutions per site.

strain are quite different. First, field canker inspectors are asked to report any *Xac*-A^W suspect citrus and submit a sample if they find CC on Key/Mexican lime but not on other susceptible citrus cultivars. Indirect ELISA using MAb A1 antibody can easily separate *Xac*-A^W from *Xac*-A strains since MAb A1 antibodies only react with *Xac*-A strains. The two strains also differ in fatty acid profiling, and each can be differentiated by computer comparisons of profiles stored in a library. Amplification of a DNA fragment by PCR, using primers developed for amplification of a DNA fragment from *Xac*-A strains (25), did not occur with DNA from the *Xac*-A^W strains, but did with strains of *Xac*-A. The two groups of strains also differ in profiles of DNA fragments generated by rare-cutting restriction enzymes and separated by pulsed-field electrophoresis. They can also be distinguished using the BOX and ERIC primers used in rep-PCR (10). In our study, there was no clear differentiation among the strains using the Biolog GN microplate technique (Biolog Inc.). Although Biolog metabolic profiles of xanthomonads can be useful at the species level, their application for ranking intraspecific groups of strains is very limited (48). The simplest and most reliable method used to distinguish two strains is to test their pathogenicity on grapefruit plants. The different characteristics listed above are based on strains that occur in Florida at present. The usefulness of these characteristics could change if new strains of citrus canker–causing bacterium unknown to the world are introduced into or appear in Florida.

Presently, three pathovars of *X. axonopodis*—*citri*, *aurantifolii,* and *citrumelo*—are recognized and accepted (18). The pathovar classification is defined to differentiate at the infrasubspecific levels strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more hosts (13). However, the strains in each of the three pathovars have pathogenic as well as genetic similarities, whereas pathogenic and genetic dissimilarities occur between them (14,17,19,22, 24,33,41,48,49). Based on the pathogenic and genetic characteristics of the xanthomonads pathogenic to citrus plants, the classification of strains belonging to *Xac*-A* and *Xac*-A^W groups presents a dilemma. Both *Xac*-A* and *Xac*-A^W strains have a similar host range to pathovar *aurantifolii*, but genetic similarities to pathovar *citri*. Therefore, it does not seem proper to classify these strains in pathovar *citri* because of pathogenicity differences, and it does not seem proper to place them in pathovar *aurantifolii* because of genetic differences. Furthermore, their distinct pathogenicity cannot be recognized at the pathovar level because they would be subdivisions of an already classified pathovar. For the present, the precedent established by Vernière et al. (49) to use the *X. axonopodis* pv. *citri* A* designation for similar strains from Southwest Asia can be continued, and the designation for the unique strains in Florida can be designated as *X. axonopodis* pv. *citri* A^W. This requires that the Asiatic canker strains be designated as *X. axonopodis* pv. *citri* A. This type of designation then places genetic characteristics superior to pathogenicity, but does recognize pathogenic differences within genetically similar organisms.

The *Xac*-A^W strains studied so far in Florida reacted very uniformly in all tests and are probably clones and may have originated from a single introduction into Florida. Based on BOX and ERIC PCR analyses (10), the strain may have been introduced from one of the areas where *Xac*-A* strains were discovered. On the other hand, the *Xac*-A* strains were not genetically uniform (10), and they originated from several countries in Southeast Asia and may represent a larger population of the bacteria in the region (10,49). It may be that the *Xac*-A^W strains are members of a larger group of strains that include both *Xac*-A* and *Xac*-A^W. It was speculated that both *Xac*-A^W and *Xac*-A* strains originated in India (36,49). It would be interesting to

Table 3. Percent DNA similarity matrix for strains of xanthomonads pathogenic on citrus[a]

	% DNA similarity					
	Probe DNAs					
Tester DNAs	**X0000055**	**X0000058**	**X0012875**	**ATCC49118**	**ATCC51306**	**ATCC49120**
	Xanthomonas axonopodis pv. *citri* group					
1) X0000055 (*Xac*-A^W)	100.0	93.4	88.2	71.9	40.1	35.8
		8.6	2.5	1.4	0.1	0.6
2) X0000058 (*Xac*-A^W)	85.1	100.0	98.5	70.7	28.8	38.7
	1.7		2.9	5.0	3.4	1.5
3) X0012875 (*Xac*-A^W)	81.9	85.1	100.0	76.9	35.6	64.2
	1.6	2.8		9.8	2.8	1.0
4) ATCC49118 (*Xac*-A)	81.2	89.5	94.1	100.0	35.2	44.3
	2.5	2.1	5.3		NA	0.4
5) XC205 (*Xac*-A*)	85.7	90.3	82.0	81.7	30.9	37.5
	4.8	7.7	3.6	10.8	7.3	3.1
6) XC406 (*Xac*-A*)	81.4	86.1	79.3	77.4	36.3	37.4
	3.3	6.1	1.3	4.7	0.3	0.5
				X. axonopodis pv. *aurantifolia* group		
7) ATCC51306 (B-strain)	49.1	34.6	47.6	45.0	100.0	41.2
	1.4	3.7	6.7	0.6		3.6
8) ATCC51302 (B-strain)	41.1	44.5	50.6	46.0	84.7	33.9
	1.6	9.8	4.8	4.1	3.1	1.9
					X. axonopodis pv. *citrumelo*	
9) ATCC49120 (CBS[b])	35.7	35.3	56.5	33.3	24.0	100.0
	2.6	1.1	4.2	0.8	0.8	
10) XCC3048 (CBS)	51.7	50.0	43.3	48.3	28.1	76.5
	11.8	6.2	16.2	2.3	1.8	3.2
			X. axonopodis pv. *vasculorum*			
11) ATCC35938						
	25.8	19.1	19.5	13.9	22.1	28.1
	3.9	8.5	0.3	5.5	1.3	1.4

[a] Boxes contain percent DNA similarity values consistent with species-level taxons (% DNA similarity greater than or equal to 70%). Standard error among observations is indicated below each value.

[b] CBS = citrus bacterial spot.

collect a group of strains from Key/Mexican lime in India to validate this speculation.

Based on the preliminary results, a group of plant pathologists from Florida recommended in February 2001 that the Wellington group of strains be characterized as a unique group of strains of *X. axonopodis* pv. *citri* and that all of its host plants, Key/Mexican lime and alemow, be removed within 579 m (1,900 feet) of a diseased plant.

Identification of the *Xac*-A^W strains is essential in Florida because the limited host range of *Xac*-A^W is a sound reason for the eradication programs to have different policies for the two strains. Field diagnosis of citrus canker would be difficult on Key/Mexican lime if *Xac*-A and *Xac*-A^W occurred in the same area because the strains cause identical canker syndromes on that variety. In order to determine if both strains exist in the same area, the pathology teams surveying for the possible presence of *Xac*-A^W had been active for a period of 6 months in about 54 square miles in a 6-mile-wide zone across southern Broward County and northern Dade County where citrus canker was present. Eight diseased Key/Mexican limes were identified on the properties where citrus canker was not found on other citrus varieties nearby. All eight samples collected from those Key/Mexican limes tested positive for *Xac*-A using ELISA and pathogenicity tests, indicating that *Xac*-A^W had not spread to the adjacent areas where *Xac*-A has been present since its introduction. Routinely, citrus canker samples of Key/Mexican lime from distant sites where no other nearby citrus varieties were infected have been tested for possible presence of *Xac*-A^W, but none of them so far have tested positive for citrus canker caused by *Xac*-A^W. For these reasons, we believe that *Xac*-A^W occurs only in the areas where it was initially detected. In the event that a sample is suspected to be an *Xac*-A^W infection, several tests such as a pathogenicity test on Duncan grapefruit and ELISA using MAb A1 antibody can be used to distinguish *Xac*-A from *Xac*-A^W.

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